Beneficial Effects of Penicillamine Treatment on Hereditary Avian Muscular Dystrophy

TA-HSU CHOU, EDWARD J. HILL, EDWARD BARTLE, KEVIN WOOLLEY, VIRGIL LEQUIRE, WILLIAM OLSON, ROBERT ROELOFS, and JANE H. PARK

From the Departments of Physiology and Pathology, and the Neuromuscular Disease Center, Vanderbilt University Medical School, Nashville, Tennessee 37232

ABSTRACT Hereditary muscular dystrophy in chickens of the New Hampshire strain was treated with penicillamine from the 9th day after hatching to the 425th day. The adult maintenance dose for males was 50 mg/kg per day and for females, 13–65 mg/kg per day. In avian dystrophy, deterioration of the muscle fibers is evidenced in the 2nd mo by an inability of the birds to rise after falling on their backs and by a progressive rigidity of the wings. The drug delayed the onset of symptoms and partially alleviated the debilitating aspects of the disease. Penicillamine produced three major improvements: (*a*) better righting ability when birds were placed on their backs; (*b*) greater wing flexibility; (*c*) and suppression of plasma creatine phosphokinase activity. The results are statistically analyzed and discussed in relationship to Duchenne dystrophy. Normal birds were not affected by penicillamine as judged by these parameters.

The rationale for using penicillamine, a sulfhydryl compound with reducing properties, was (*a*) to attempt to protect essential thiol enzymes in the anabolic and glycolytic pathways against inactivation and (*b*) to prevent collagen cross-linking and deposition in muscle. Although the precise mechanism of drug action has not been determined, the possible role of penicillamine in mitigating the symptoms of genetic dystrophy in man is under consideration. Further, penicillamine may have a more generalized application in the prevention of contractures in a variety of neuromuscular disorders.

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INTRODUCTION

This paper reports the results of our five-yr study on penicillamine treatment of hereditary muscular dystrophy in chickens. Genetic muscular dystrophy in the chicken may be considered as a model for inherited dystrophic diseases in humans (2). In avian dystrophy there is progressive destruction of muscle fibers with necrosis, phagocytosis, and replacement of muscle with adipose and collagenous connective tissue. During the process there is an elevation of serum levels of muscle enzymes, such as creatine phosphokinase (CPK),[1] aldolase, and serum glutamic oxaloacetic transaminase. The onset of symptoms in dystrophic chickens occurs in the 2nd mo after hatching. Chickens are unable to right after falling on their backs, and their wings become excessively stiff and eventually cannot be elevated beyond a horizontal plane. Penicillamine treatment partially alleviated the symptoms of the avian disease by improving the righting ability, producing greater flexibility of the wings, and suppressing plasma CPK levels. The relationship of these findings to human dystrophies and therapy will be considered in the Discussion.

The rationale behind penicillamine treatment was twofold: (*a*) to alter the intracellular redox status, thereby preventing oxidation of critical sulfhydryl groups of proteins; and (*b*) to decrease the formation of cross-linked, insoluble collagen in muscle. Penicillamine was selected as a suitable drug for a program with this rationale because it is relatively stable to auto-oxidation and metabolic degradation (3). Moreover, penicillamine blocks collagen cross-linking by inhibiting the formation of Schiff's base intermediates and by cleaving inter-

[1] *Abbreviation used in this paper:* CPK, creatine phosphokinase.

molecular bonds (4, 5). Further studies are in progress to determine the mechanism of drug action.

METHODS

Chickens and maintenance. Dystrophic eggs and chickens were obtained as a gift from Dr. Louis Pierro, Department of Animal Genetics University of Connecticut at Storrs. Dystrophic chickens were of *am/am* mutation, originally seen in New Hampshire chickens and crossed with normal Leghorn stock (2). Normal white Leghorn chickens were purchased from Smith Blanton Co., Nashville, Tenn. The birds were maintained on Purina Startena (Ralston Purina Co., St. Louis, Mo.). When an attempt was made to switch the dystrophic flock to Eggena or Layena, the new diet produced a deleterious effect on the general health of the birds. Water was supplied in plastic troughs to avoid excessive metal intake and the possible removal of free penicillamine in the body by formation of a chelated complex.

Grouping of chickens. Young chicks were matched in pairs according to sex, body weight, and general health. The paired chickens were separated into untreated and penicillamine-treated groups. Detailed data are reported for two separate studies: exp. A is a double-blind study on normal and dystrophic chickens, followed for 105 days; exp. B is a long-term experiment with a larger group of dystrophic chickens under treatment for 425 days.

Penicillamine treatment. Penicillamine (D-β,β dimethyl cysteine) was obtained from Merck Sharp & Dohme (Division of Merck & Co., Inc., Rahway, N. J.) as Cuprimine capsules. 32 capsules (250 mg/capsule) were opened and the powder was suspended in 40 ml of water. The suspension was heated in a water bath between 40 and 50°C for 15 min and the capsule filler was removed by filtration. The final penicillamine solution, 200 mg/ml as assayed by 5,5'-dithiobis-(2-nitobenzoic acid) titration (6), was stored at −20°C for no longer than 2 mo. The capsules of penicillamine were used rather than the pure compound so that this work would be comparable to any future clinical experiments.

Starting on the 9th day after hatching, chicks were fed penicillamine or water daily through a tuberculin syringe without a needle. After 1 mo, a Schwarz/Mann automatic biopette was employed (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). The feeding process had to be done slowly to prevent spitting, choking, or flooding of the lungs. In the first 3 or 4 mo, the dosage of penicillamine was determined by body weight and the general health of the birds. For chickens with diarrhea, vomiting, lethargy, or sudden weight loss, the dose of penicillamine was decreased. Average daily dosages are recorded in Figs. 1 and 4.

Tests for measuring the progress of the disease

Four parameters were used to examine the effects of penicillamine: body weight, righting ability, wing apposition, and CPK levels in the plasma.

Righting ability. Normal chickens can right themselves immediately when placed on their backs on a flat surface. Dystrophic birds usually start to show difficulty in righting after about 30–40 days of age. Since righting ability is determined by strength and wing flexibility, it is one of the best means to measure the progress of avian dystrophy.

Righting ability was tested as follows: the bird was placed on its back, and if it could not rise it was allowed to flap until exhausted. Two righting trials were done consecutively; this was followed by the measurements of body weight and wing apposition. Then two more trials for righting were repeated. The number of successful trials was recorded for each chicken. The righting tests, wing measurements, and weighings were carried out twice a week.

Wing apposition. The wings of a normal chicken can be raised passively without resistance until they touch each other. However, the wings of dystrophic chickens begin to show some inflexibility at approximately 30 days. The distance between the two wings was measured from the styloid processes of the radii with a plastic ruler. Distances were measured independently by five individuals to check on the accuracy of the method and were found to be readily reproducible.

CPK levels in plasma. Blood (1.0–2.0 ml) was drawn from the brachial vein, heparinized, and spun at 2,000 rpm for 10 min. Plasma samples were stored at −20°C until the assays were performed. CPK activity was measured by a slightly modified method of Sigma Chemical Co. (St. Louis, Mo.) as described in Sigma Technical Bulletin No. UV-40, revised February, 1967, and reissued August, 1971. Sigma units were converted to international units by comparing our analyses of CPK standards from Sigma or plasma samples with results obtained in the Vanderbilt Clinical Laboratory by the automatic procedure of Technicon (N-76) (Technicon Instruments Corp., Tarrytown, N. Y.).

RESULTS

Experiment A

Design of exp. A. Eggs from normal and dystrophic chickens were hatched in our laboratory. The birds were randomly separated into four groups: untreated normals, penicillamine-treated normals, untreated dystrophics, and penicillamine-treated dystrophics. Since penicillamine has a very strong odor and cannot be disguised, a modified double-blind study was designed. Birds were administered either penicillamine or a water placebo by one investigator, who regulated the dose of the drug as described in Methods. The righting tests and wing apposition measurements were made independently by two other investigators who had no knowledge of the grouping of the chickens. This double-blind experiment insured that the measurements were objective and unbiased.

During the experiment five birds died. Two treated males (one normal and one dystrophic) died as a result of improper administration of penicillamine and flooding of the lungs. Two untreated birds (a normal female and a dystrophic male) died on the 45th day due to excessive anesthesia during muscle biopsy. Another bird died of unknown cause (a normal, treated female), and autopsy showed all organs were normal.

Body weight. Penicillamine treatment initially reduced the growth rate of both normal and dystrophic chickens. On the 105th day, the body weights of treated, dystrophic hens and roosters were approximately 15% lower than their untreated counterparts (Fig. 1). How-

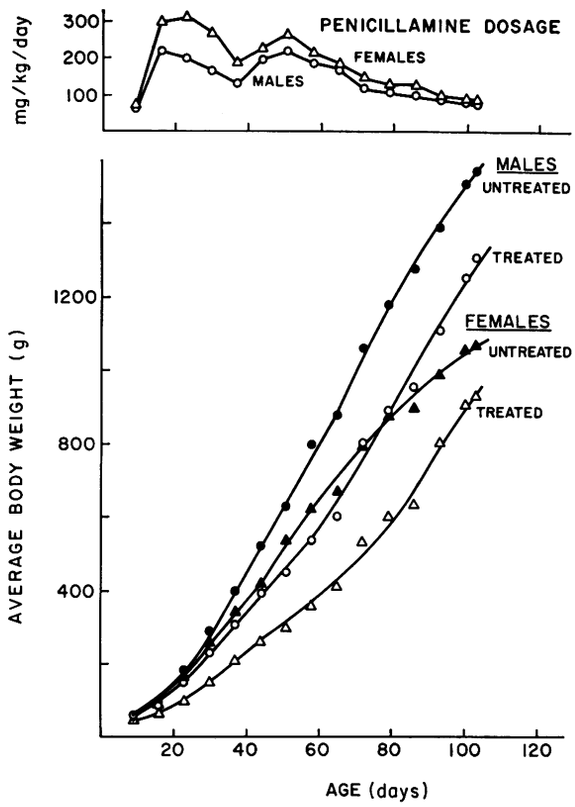

FIGURE 1 Growth curves in exp. A. Body weights were measured twice a week, but for simplification of the graphs, only the points on the first day of the week were plotted. This also applies to Figs. 2 and 3.

ever, exp. B (Fig. 4) showed that the weights of treated and untreated females were equal in the 4th mo, and the weights of males in the 5th mo.

Normal males had essentially the same average body weight as the dystrophic males for 105 days. This was also true for the normal and dystrophic females. During growth, penicillamine treatment suppressed the weight of the normal and dystrophic chickens by approximately the same percentages.

Righting. After about 2 or 3 wk, untreated, dystrophic birds began to lose their ability to right (Fig. 2). Males lost the righting ability earlier and at a faster rate than females. From 40 days onward, marked effects of penicillamine were observed. Untreated males were unable to right whereas penicillamine-treated males could right themselves on the average more than 50% of the time. Penicillamine-treated females could right three or more times, or an average of about 80–90% successful trials. The data were statistically analyzed, and the standard errors of the mean graphed in Fig. 2. Using Student's *t* test, penicillamine-treated males showed significantly better righting during the last 2½ mo of the experiment. *P* values of less than 0.05 were observed for measurements on 17 out of 21 days. For the females, a statistically significant difference between treated and untreated birds was seen in the last month on 6 out of 10 trial days.

Normal chickens, both treated and untreated, were able to right themselves successfully on all four trials throughout the entire experiment. Therefore, the data were not plotted in Fig. 2.

Wing apposition. During the 1st mo, the distance between the raised wings was zero in all dystrophic chickens, indicating that the wings could be easily approximated (Fig. 3). Subsequently, untreated males showed the greatest rigidity, with an average of 5.2 inches between the wings by the 3rd mo. Penicillamine treatment partially alleviated the stiffness as evidenced by the final distance of 2.0 inches in the treated males. The treatment produced a statistically significant improvement in wing flexibility during the last month on 8 out of 9 testing days, as indicated by *P* values less than 0.05.

Untreated females were not as severely affected as their male counterparts, and after 100 days females

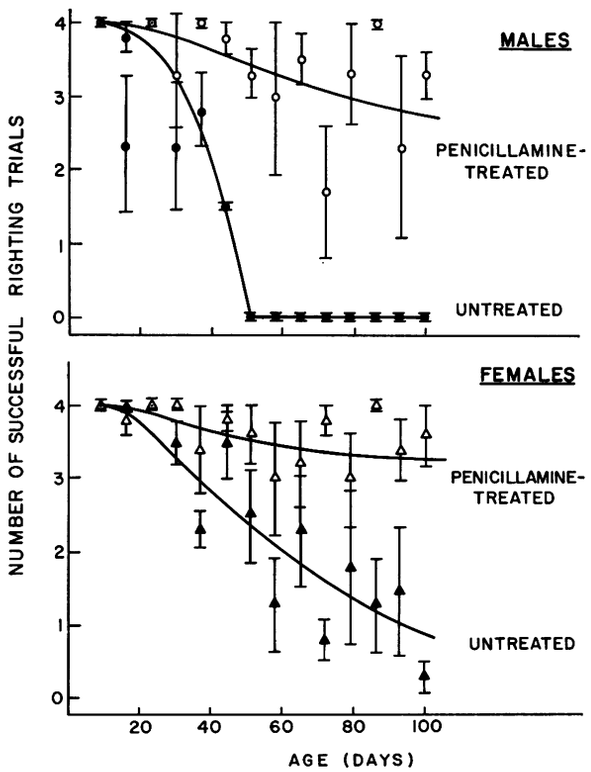

FIGURE 2 Righting ability in exp. A. The number of successful rightings out of a total of four trials (see Methods) were recorded over a period of 105 days. The top panel shows the average values for dystrophic males and the bottom panel, the data for dystrophic females. The length of the vertical bar represents two SEM.

showed an average distance of 3.0 inches. The small reduction in apposition distance to 2.2 inches around 70 days is not understood at present. After the 2nd mo, penicillamine treatment improved the wing flexibility of the females to the point of almost perfect apposition. By Student's *t* test, the mean wing distance of treated and untreated hens were statistically different in all 12 trials after the 60th day.

The wings of all normal chickens were easily approximated throughout the entire experiment.

CPK levels. The plasma CPK activity of every chicken was analyzed on the 105th day (Table I). Untreated dystrophic males and females had high CPK values of approximately 25,000 IU/liter, and penicillamine suppressed the CPK levels to about 8,000 IU/liter. Statistical analysis of the treatment showed a *P* value of 0.03 for males and 0.001 for females. This suppression did not result from an inhibition of plasma CPK by the drug. Added penicillamine at concentrations as high as 50 mM had no inhibitory effect on the CPK assay system. There was no significant difference in the CPK activity of untreated and penicillamine-treated normal chickens.

TABLE I
Plasma CPK Levels in Experiment A

Type of bird	Male	Female
	IU/liter	
Dystrophic, untreated	25,500±5,000 (3)	24,600±2,500 (4)
Dystrophic, treated	7,800±1,700 (3)	8,800±1,200 (5)
P	0.03	0.001
Normal, untreated	1,800±240 (5)	1,600±90 (5)
Normal, treated	1,900±100 (5)	1,400±40 (4)
P	NS	NS

CPK levels were determined for all chickens on the 105th day, as described in Methods. Mean values±SEM are presented, and the number of chickens in each group is shown in parentheses. The probability value (*P*) was determined from Student's *t* test.

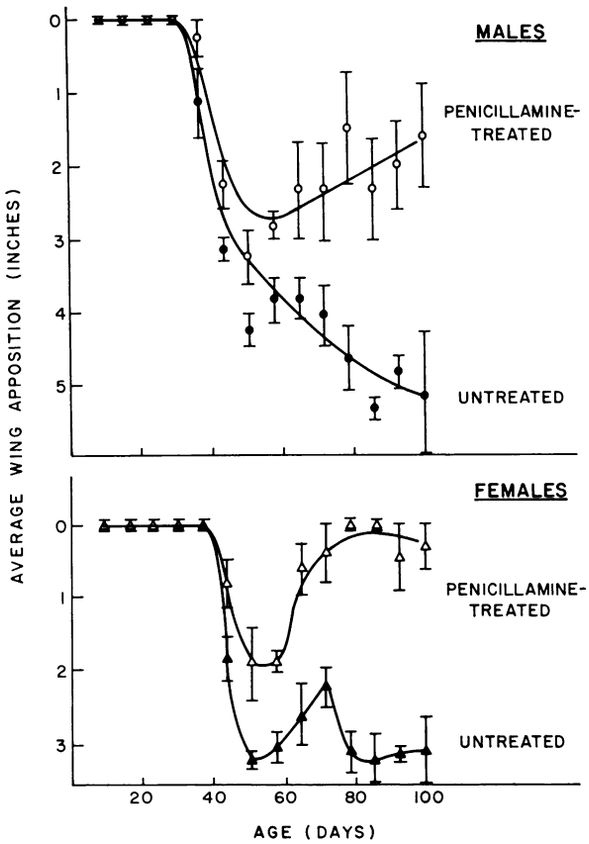

FIGURE 3 Wing apposition in exp. A. The distance between the wings was measured as described in Methods. Each point represents the average distance, and the length of the vertical line is 2 SEM.

The chickens of exp. A were not evaluated beyond the 4th mo, as they were sacrificed to obtain tissue samples for future histochemical and chemical analyses.

Experiment B

General physical condition and penicillamine treatment. Newly hatched, dystrophic chicks were shipped by air from Connecticut. Chicks were matched and divided into untreated and penicillamine-treated groups. There were 26 chicks in the untreated group (9 male, 17 female) and 27 in the penicillamine-treated group (11 male and 16 female). The dose of penicillamine was administered daily and regulated as described in Methods. After 280 days, there were 22 untreated chickens (8 male, 14 female) and 20 penicillamine-treated birds (11 male, 9 female). The increased death rate of female chickens in the 2nd and 4th mo was probably caused by improper feeding of penicillamine by new assistants. Chickens in apparent good health died suddenly without symptoms within 12 h of the penicillamine feeding. Autopsy (gross and microscopic) showed that death was due to aspiration pneumonia. All organs other than the lungs were normal. The three chickens that expired in this manner had essentially mean values for both righting and wing measurements.

The starting dose of penicillamine in exps. A and B was approximately 200 mg/kg per day, and by the 9th or 12th wk, the amount was lowered to 140 mg/kg per day (Figs. 1 and 4). Young birds had a greater tolerance for the drug than adult birds. The maintenance dose for males was 50 mg/kg per day. This dosage is no larger than that used for treatment of human cystinuria. The females in exp. B did not tolerate this level and were progressively reduced to 32 and ultimately to 13 mg/kg per day. The necessity for this reduction was unexpected, as females in two

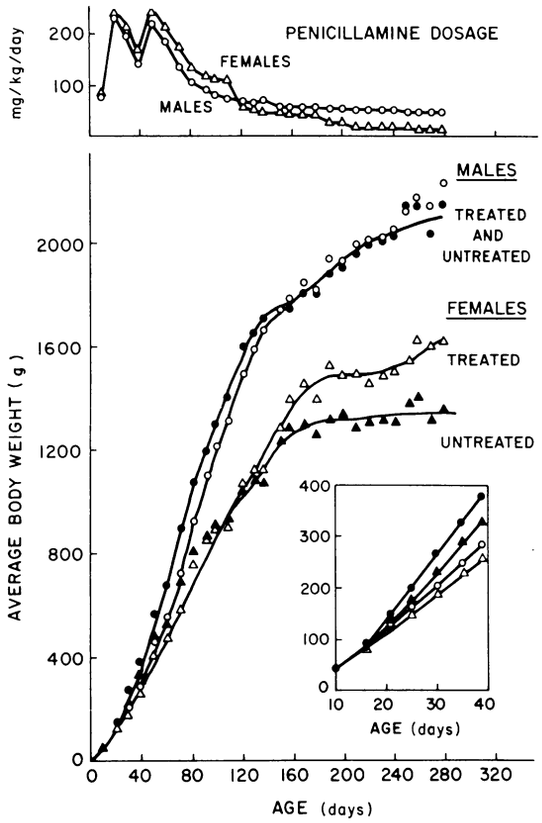

FIGURE 4 Growth curves in exp. B. The points plotted in this graph represent the average weights closest to every 10th day, although the actual data were collected twice a week. This also applies to Figs. 5 and 6.

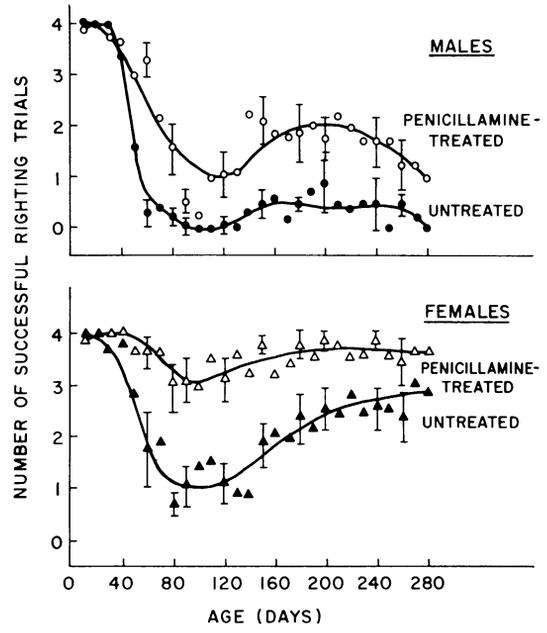

FIGURE 5 Righting ability in exp. B. The number of successful rightings out of a total of four trials were recorded and plotted as the average values. The length of the vertical line represents 2 SEM.

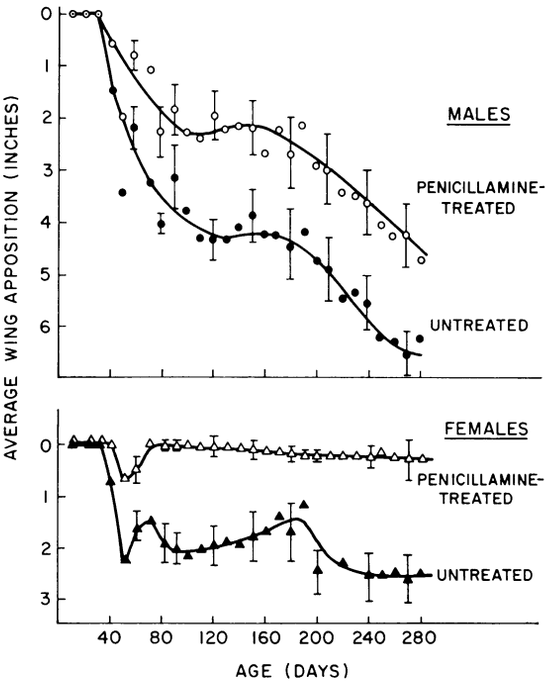

FIGURE 6 Wing apposition in exp. B. Measurements were made as described in Methods and plotted in a manner analogous to Fig. 5.

preliminary experiments tolerated doses as high as 65 mg/kg per day.

Body weight. 10 days after hatching, the average body weight in all subgroups was about 53 g (Fig. 4). Up to 50 days of age, the penicillamine-treated group, both male and female, had a 10–20% lower body weight. By 160 days, penicillamine-fed males and untreated males had the same average body weight and remained equal thereafter. At about 120 days, penicillamine-fed females became equal in weight to the untreated females, and by 180 days were 10% heavier.

Righting. After 30 days, untreated birds began to lose the ability to right (Fig. 5). At two mo, untreated males had an average of only 0.4 successful trials (10%). Penicillamine-treated males showed a much slower decline and were able to right successfully on the average more than three times (80%). By 150 days, the number of successful righting trials for treated males plateaued at 2.0 or 50%. Approximately the same value was observed for untreated females. However, penicillamine-treated females had an almost perfect score throughout the entire 280 days and could

right themselves 90% of the time. After 200 days, an unusual recovery of righting ability by untreated females was noted as peculiar to this particular group of birds. Roosters, both untreated and treated, never showed signs of recovery during this 3-mo period. Testing and treatment were continued until day 425, and the righting values remained constant. In a detailed analysis from the 50th through the 210th day, 91% of the trials showed statistically significant improvement for the treated hens and 54% of the tests for treated roosters. From 50 through 80 days, the roosters showed statistically better righting on each trial day.

Wing apposition. During the 1st mo the distance between the raised wings was zero in all chickens (Fig. 6). After 70 days, the subgroups began to show distinct differences, and by 200 days separated into three categories: (*a*) untreated males showed the greatest rigidity with an average of 4.9 inches between the wings; (*b*) penicillamine-fed males and untreated females had an average distance of 2.0–2.5 inches between the wings; (*c*) and the wings of penicillamine-fed females remained very flexible with an average distance less than 0.5 inches during the entire period. From the 40th to the 280th day, penicillamine treatment produced significant improvement on 97% of the test days with females and 75% of the test days with males as analyzed by Student's *t* test. Wing distances remained constant from day 280 to 425.

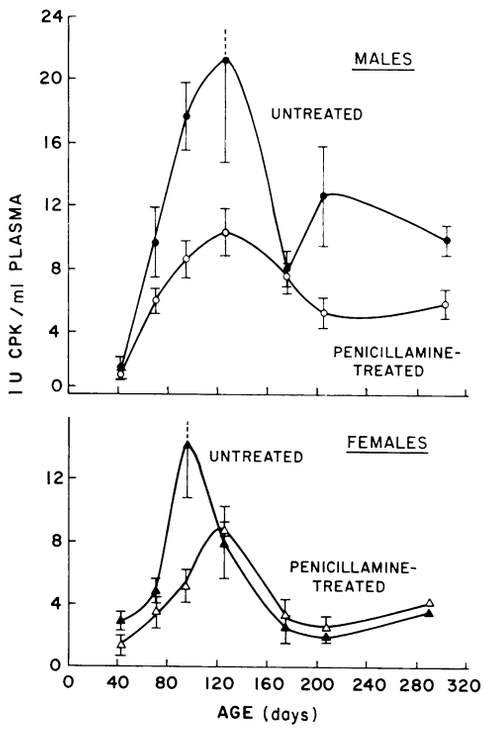

FIGURE 7 Plasma CPK levels in exp. B. CPK analyses were performed as described in the text. On any given day, each point is the average CPK value for five dystrophic chickens.

CPK levels. For the plasma CPK analysis on any given day, five males and five females were randomly selected from the untreated and penicillamine-treated groups. The first analyses of the males on the 43rd day showed low values of about 1,500 IU/liter (Fig. 7). The CPK of untreated males rose to 17,600 on the 95th day and reached a maximum of 21,300 on 127th day. Penicillamine treatment suppressed these values to approximately half, 8,700 on the 95th day and 10,400 on the 127th day. There was also a lower plasma CPK level in the penicillamine-treated males subsequently on days 207 and 303. Farrell et al. have observed considerable variation in the time of CPK peaking and decline in individual chickens (7). The unexpected drop in CPK value for the untreated male on the 165th day may reflct this type of variability. In a separate experiment, another group of untreated roosters had an average CPK value on the 150th day of 18,000, well above the treated level. Penicillamine treatment reduced the variability in both males and females, as indicated by the smaller standard error of the mean during the peak of CPK activity (Fig. 7).

From the earliest analyses on days 43 and 76, untreated females had substantially higher CPK activities than the treated females. The peak of 14,000 IU/liter on the 95th day was lowered by penicillamine treatment to 5,200. By the 127th day the CPK activities for both untreated and treated females were essentially identical and remained so until the 280th day. According to Student's *t* test, the means were significantly different during the CPK peak at the 95th day for both hens and roosters. In normal chickens, the CPK levels are essentially constant for the first 170 days (7).

DISCUSSION

Therapeutic evaluation. Penicilliamine improved the dystrophic chickens as evidenced by righting ability and wing flexibility. The improved righting was not related to body weight but correlated with greater flexibility of the wings, as indicated in Figs. 2, 3, 5, and 6. Comparison of individual chickens in any given category also confirmed this finding. However, in the terminal stages, some birds were so weak that they could not turn over despite very flexible wings.

Since the rigidity of the wings may be analogous to the contractures of human dystrophies, the effect of penicillamine on flexibility may be of clinical interest. Prevention of contractures in dystrophic patients might facilitate arm or hand movments and also prolong the period of walking.

Another observation of potential clinical interest was that earlier administration of penicillamine produced

more substantial improvement. In a separate experiment, penicillamine treatment was started on the 27th day after hatching rather than the 9th day, and the improvement in both righting ability and wing flexibility was less pronounced.

Mechanism of therapeutic action. Improved muscle function in righting tests is compatible with the hypothesis that penicillamine protects critical sulfhydryl enzymes by maintaining a suitable intracellular redox state. Sulfhydryl compounds, such as penicillamine and *N*-acetyl-cysteine, penetrate cells and increase protein sulfhydryl levels (8). The catalytic site of glyceraldehyde-3-phosphate dehydrogenase has an exceptionally reactive sulfhydryl group required for high energy phosphate production (9, 10). A number of other glycolytic enzymes, including CPK, also require sulfhydryl groups for maximal activity (11). Penicillamine in vivo could protect these enzymes, either by disulfide interchange or by chelation with divalent metal ions that cause oxidation of sulfhydryl groups. In dystrophic chickens, the white muscle fibers depend primarily on the glycolytic pathway for ATP and are more severely affected than red muscle fibers (2). These facts, however, are not sufficient to confirm the importance of sulfhydryl enzymes in the mechanism of drug action.

The effects of penicillamine on joint flexibility may be related to its action on collagen. This drug blocks the formation of new insoluble collagen, inhibits cross-linking, and degrades a certain fraction of recently synthesized insoluble collagen (4, 5). These properties may alleviate tendon rigidity and infiltration of muscle by collagen. The greater improvement in flexibility associated with the earlier treatment of the chicks may also be explained by the work of Nimni et al. (5), who found that the amount of degradation of preformed collagen in penicillamine-treated rats was greater in younger animals. The collagen extracted from younger rats was also more readily solubilized by penicillamine in vitro.

The suppression of plasma CPK by penicillamine may reflect a beneficial effect in preventing membrane damage and enzyme "leakage". Protein sulfhydryl groups and structural lipids are thought to be necessary for regulated permeability and integrity of membranes (12–14). Metallic cations, such as copper or ferrous iron, catalyze the oxidation of protein sulfhydryl groups to disulfide bonds and of unsaturated lipids to peroxides, thereby impairing membrane function. Penicillamine may stabilize membranes by preserving sulfhydryl groups and chelating divalent metals (8). Penicillamine, like vitamin E or cysteine, could also act as a chain breaker to decompose free radicals and peroxides of unsaturated fatty acids that lead to disruption of muscle membranes (15) and lysosomes (13, 16, 17).

Currently the neural hypothesis of the etiology of muscular dystrophy is receiving considerable attention. In this regard, sulfhydryl groups in the proteins of synaptic membranes may be important in neurotransmitter release as it relates to myopathies. Thiol-oxidizing agents, such as diamide, increase the number of disulfide bonds in the membrane proteins of the end-plate with concurrent release of acetylcholine (18, 19). Excess acetylcholine has been shown to induce a myopathy in rat muscle with histochemical characteristics similar to Duchenne dystrophy (20). The effect of diamide can be counteracted by reducing compounds, such as glutathione (18, 19). Studies are currently underway to investigate further the mechanisms of therapeutic action of penicillamine in dystrophic chickens.

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